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Functional Characterization of Ost3p. Loss of the 34-kD Subunit of the *Saccharomyces cerevisiae* Oligosaccharyltransferase Results in Biased Underglycosylation of Acceptor Substrates

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Abstract. Within the lumen of the rough endoplasmic reticulum, oligosaccharyltransferase catalyzes the en bloc transfer of a high mannose oligosaccharide moiety from the lipid-linked oligosaccharide donor to asparagine acceptor sites in nascent polypeptides. The *Saccharomyces cerevisiae* oligosaccharyltransferase was purified as a heteroligomeric complex consisting of six subunits (α-ζ) having apparent molecular masses of 64 kD (Ost1p), 45 kD (Wbp1p), 34 kD, 30 kD (Swp1p), 16 kD, and 9 kD. Here we report a structural and functional characterization of Ost3p which corresponds to the 34-kD γ-subunit of the oligosaccharyltransferase. Unlike Ost1p, Wbp1p, and Swp1p, expression of Ost3p is not essential for viability of yeast. Instead, *ost3* null mutant yeast grow at wild-type rates on solid or in liquid media irrespective of culture temperature. Nonetheless, detergent extracts prepared from *ost3* null mutant membranes are twofold less active than extracts prepared from wild-type membranes in an in vitro oligosaccharyltransferase assay. Furthermore, loss of Ost3p is accompanied by significant underglycosylation of soluble and membrane-bound glycoproteins in vivo. Compared to the previously characterized *ost1-1* mutant in the oligosaccharyltransferase, and the *alg5* mutant in the oligosaccharide assembly pathway, *ost3* null mutant yeast appear to be selectively impaired in the glycosylation of several membrane glycoproteins. The latter observation suggests that Ost3p may enhance oligosaccharide transfer in vivo to a subset of acceptor substrates.

ASPARAGINE-linked glycosylation of proteins within the lumen of the rough endoplasmic reticulum (RER)[1] occurs via a highly conserved pathway in all eukaryotic organisms (Herscovics and Orlean, 1993; Kornfeld and Kornfeld, 1985). An increasing body of evidence articulates the crucial role of N-linked oligosaccharides in the physicochemical properties and biological function of many secreted and integral membrane glycoproteins (Varki, 1993). The oligosaccharyltransferase (OST) catalyzes the en bloc transfer of a core unit of 14 saccharides ($Glc_3Man_9GlcNAc_2$) from a dolichol pyrophosphate donor, onto the nitrogen of an asparagine side chain in a Asn-X-Ser/Thr consensus sequon (Kornfeld and Kornfeld, 1985). Studies in *Saccharomyces cerevisiae* have provided important information concerning the biosynthesis of asparagine-linked oligosaccharides (Herscovics and Orlean, 1993; Kukuruzinska et al., 1987). In particular, the *alg* (*a*sparagine-*l*inked-*g*lycosylation) mutants have been invaluable in defining the assembly pathway for the dolichol-linked oligosaccharide donor and in identifying the individual glycosyltransferases responsible for the sequential transfer of monosaccharides onto dolichol phosphate (Huffaker and Robbins, 1982; Kukuruzinska et al., 1987).

Insight into the membrane organization and subunit composition of the oligosaccharyltransferase was first disclosed upon purification of the canine and avian oligosaccharyltransferase as protein complexes consisting of ribophorin I (66 kD), ribophorin II (63 kD), and OST48 (48 kD) (Kelleher et al., 1992; Kumar et al., 1994). As shown previously for ribophorins I and II (Crimaudo et al., 1987; Harnik-Ort et al., 1987), protein sequence analysis and protease accessibility studies revealed that OST48 is oriented so that the majority of the polypeptide is located within the ER lumen (Silberstein et al., 1992). The protein sequence of OST48 is homologous to Wbp1p (Silberstein et al., 1992), a 45-kD yeast ER membrane protein shown to be required for in vivo and in vitro expression of oligosaccharyltransferase activity (te Heesen et al., 1992). Subsequently, the oligosaccharyltransferase of *S. cerevi-*

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Abbreviations used in this paper: CPY, carboxypeptidase Y; DPAP B, dipeptidyl aminopeptidase B; Endo H, endoglycosidase H; OST, oligosaccharyltransferase; PVDF, polyvinylidene difluoride; RER, rough endoplasmic reticulum; SRP, signal recognition particle; TM, tunicamycin.

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siae was purified as a heteroligomer consisting of Wbp1p, Swp1p and four additional subunits of 62/64, 34, 16, and 9 kD (Kelleher and Gilmore, 1994). The 30-kD Swp1 protein, which was known to form complexes with Wbp1p in the yeast RER and to be required for N-linked glycosylation in vivo (te Heesen et al., 1993), was found to be homologous to the carboxy terminal half of ribophorin II (Kelleher and Gilmore, 1994). The heterogeneously glycosylated 62/64-kD subunit of the yeast OST was recently shown to be homologous to ribophorin I (Silberstein et al., 1995). Like *WBP1* and *SWP1* (te Heesen et al., 1991, 1993), the *OST1* gene that encodes the 62/64-kD subunit is an essential yeast gene (Silberstein et al., 1995).

Asparagine-linked glycosylation of proteins occurs during or shortly after transport of acceptor sites into the lumen of the endoplasmic reticulum (Rothman and Lodish, 1977). One poorly understood aspect of oligosaccharide transfer is that some N-X-S/T consensus acceptor sites in proteins that enter the ER lumen are not used, or are instead used with low efficiency in vivo, while other sites are quantitatively glycosylated. Glycosylation sites located within 13–amino acid residues of a membrane spanning segment are not modified in vivo, suggesting that the active site of the OST does not have access to such sites for steric reasons (Nilsson and von Heijne, 1993). A statistical analysis of non-utilized glycosylation sequons revealed a significant bias against glycosylation of sequons with the sequence N-X-T/S-P (Gavel and Von Heijne, 1990) in addition to the known lack of glycosylation at N-P-S/T sites. Although the presence of proline at the +1 or +3 position relative to asparagine can explain why many N-X-S/T sites are not used, other poorly understood features of the acceptor substrate must also influence the frequency of acceptor site modification. Several recent studies document hyperglycosylation of folding-impaired proteins in the endoplasmic reticulum (Allen et al., 1995; McGinnes and Morrison, 1994; Silberstein et al., 1995) suggesting that acquisition of protein tertiary structure and N-linked glycosylation can, under some circumstances, be competing reactions. Inhibition of disulfide bond formation in nascent glycoproteins results in efficient glycosylation of sequons that are normally not used, or used with low efficiency (Allen et al., 1995; McGinnes and Morrison, 1994). Finally, statistical analysis suggests that glycosylation sites near the carboxy terminus of proteins are used with lower efficiency in vivo compared to other consensus sites in nascent glycoproteins (Gavel and Von Heijne, 1990). Experimental support for the latter observation has been obtained by COOH-terminal truncation of the rabies virus glycoprotein (Shakin-Eshleman et al., 1993).

Once yeast homologues for each of the three mammalian subunits of the oligosaccharyltransferase had been identified, one question that arose concerned the role of the three remaining yeast subunits in oligosaccharide transfer in vivo. Conceivably, one or more of these polypeptides might perform auxiliary functions that are not absolutely required for oligosaccharide transfer. Here we describe the isolation and characterization of the *OST3* gene that encodes the 34-kD subunit of the yeast OST complex. Expression of the Ost3 protein is not essential for viability of yeast under standard laboratory conditions. However, yeast lacking the Ost3 protein underglycosylate both soluble and membrane bound glycoproteins. Surprisingly, the extent of underglycosylation of individual glycoproteins varied considerably, suggesting that the Ost3 protein increases the probability that certain glycosylation sequons will be efficiently used in vivo.

Materials and Methods

Protein Purification and Peptide Sequencing

The oligosaccharyltransferase was purified from *S. cerevisiae* as described previously (Kelleher and Gilmore, 1994) and the six subunits were resolved by denaturing gel electrophoresis and isolated as described (Silberstein et al., 1995). Tryptic digestion of the 34-kD γ-subunit, purification of tryptic peptides by reverse phase high pressure liquid chromatography, and sequencing of the amino terminus and internal tryptic peptides were performed by the Protein Chemistry Facility of the Worcester Foundation for Experimental Biology.

Isolation and Sequencing of an OST3 Genomic Clone

Two degenerate oligonucleotide primers (5′GCNTTYCARTTYTTYCARYT and 5′GGNSWRTTNGGYTTRAA) were synthesized by selecting regions with the least degeneracy (underlined) from the sequence of two tryptic peptides (AFQFFQLNNVPR and LFIFKPNSPXILDHSXXSI) and were used to amplify a yeast genomic DNA template using the PCR (Saiki et al., 1988). The amino acid residues designated X could not be unambiguously assigned from the tryptic peptide sequence data. PCR was performed in a 25 μl reaction volume with 125 pmol of primers, 2.5 U Taq DNA polymerase (Perkin Elmer Cetus Corp., Norwalk, CT) and 100 ng of *S. cerevisiae* genomic DNA. 25 amplification cycles using an automatic heating/cooler cycler (programmable thermal controller; MJ Research, Watertown, MA) were conducted as follows: 1 min at 94°C, 1 min at 40°C, and 1.5 min at 72°C. A 63-bp PCR product was recovered from a 8% polyacrylamide gel and subjected to direct DNA sequencing as described (Kusukawa et al., 1990).

A *S. cerevisiae* genomic DNA library in YEp24 was plated and two sets of nitrocellulose replicas were screened by in situ colony hybridization (Sambrook et al., 1989) using a random hexamer ^{32}P-labeled 63-bp PCR product as a probe (Sambrook et al., 1989). Positive clones were isolated and subjected to restriction analysis. A HindIII-SphI fragment (2.5 kb) from a hybridization positive genomic clone was subcloned into pGEM-4Z (Promega Biotech, Madison, WI) to generate pOST3-1. Bacterial transformation was carried out using *Escherichia coli* DH5α as described (Sambrook et al., 1989). The DNA sequence of both strands of a 1,544-bp (HindIII-BspHI) fragment containing the coding sequence of the Ost3 protein was determined using the dideoxy chain termination method (Sanger et al., 1977). DNA and protein sequence analysis was done with the MacVector (IBI, New Haven, CT) software program.

Disruption of the OST3 Gene

The plasmid pRS305ΔOST3 (Fig. 1) was constructed to replace the chromosomal *OST3* locus with the *LEU2* gene using the γ transformation procedure (Sikorski and Hieter, 1989). A 197-bp HindIII-ScaI fragment from pOST3-1 was subcloned into pRS305 which had been first digested with XhoI, blunt-ended by filling in, and then digested with HindIII to generate pRS305ΔOST3R. A 210-bp BanI-XbaI fragment derived from pOST3-1 was generated by PCR using two primers (5′GGTTATCGGCACCATTCA and 5′GCTCTAGAAGAATCATGAGATGGGCGAAG). The antisense primer contained the underlined five nucleotide extension to generate a XbaI site. The PCR fragment was digested with BanI, blunt-ended by filling in, digested with XbaI, and ligated to pRS305ΔOST3R which had been digested with NotI and blunt-ended prior to digestion with XbaI. The resulting construct, pRS305ΔOST3, was linearized at the unique SmaI site and transformed into two diploid yeast strains (PRY238 and YPH274) using a lithium acetate transformation protocol (Ito et al., 1983). Standard laboratory media were used for yeast growth and sporulation (Sherman, 1991). Leu$^+$ transformants were selected, and genomic DNA was isolated (Hoffman and Winston, 1987) to perform Southern analysis to confirm allele replacement. From each genetic background, diploid transformants were sporulated, asci were dissected and analyzed

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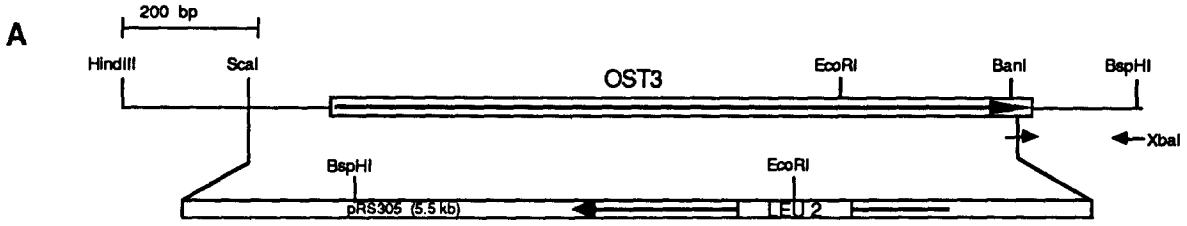

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AAGCTTTGGTTAAAACGACTAAACAAAAACTGTAAACACTTGTGTACACTATTACATATATTATTCAATAATAATTAACTGACGCAATCT      90
ATGGCACTGGCGTGGTTTCTTTTTCTTTTGTTTTCCGTGCAAGCGCGATGCAACTATATCGTCGAGGACGAATGTGTACCCGTCTTTCCG     180
CCAGACAGGTAACAAGTACTTCAATTGACTTAGAAGAAAAACATTAAGGAACTTTAACGTAGGAGCAACACAATACCAGGCACACGCTCT     270
TCGAACACTGAACCACACGCGTCCGCATCAAACTCTTCCTCCCAAACATGAATTGGCTGTTTTTGGTCTCGCTGGTTTTCTTCTGCGGCG     360
                                                  M  N  W  L  F  L  V  S  L  V  F  F  C  G

TGTCAACCCATCCTGCCCTGGCAATGTCCAGCAACAGACTACTAAAGCTGGCTAATAAATCTCCCAAGAAAATTATACCTCTGAAGGACT     450
15  V  S  T  H  P  A  L  A  M  S  S  N  R  L  L  K  L  A  N  K  S  P  K  K  I  I  P  L  K  D

CAAGTTTTGAAAACATCTTGGCACCACCTCACGAAAATGCCTATATAGTTGCTCTGTTTACTGCCACAGCGCCCGAAATTGGCTGTTCTC     540
45  S  S  F  E  N  I  L  A  P  P  H  E  N  A  Y  I  V  A  L  F  T  A  T  A  P  E  I  G  C  S

TGTGTCTCGAGCTAGAATCCGAATACGACACCATAGTGGCCTCCTGGTTTGATGATCATCCGGATGCAAAATCGTCCAATTCCGATACAT     630
75  L  C  L  E  L  E  S  E  Y  D  T  I  V  A  S  W  F  D  D  H  P  D  A  K  S  S  N  S  D  T

CTATTTTCTTCACAAAGGTCAATTTGGAGGACCCTTCTAAGACCATTCCTAAAGCGTTCCAGTTTTTCCAACTAAACAATGTTCCTAGAT     720
105  S  I  F  F  T  K  V  N  L  E  D  P  S  K  T  I  P  K  A  F  Q  F  F  Q  L  N  N  V  P  R

TGTTCATCTTCAAACCAAACTCTCCCTCTATTCTGGACCACAGCGTGATCAGTATTTCCACTGATACTGGCTCAGAAAGAATGAAGCAAA     810
135  L  F  I  F  K  P  N  S  P  S  I  L  D  H  S  V  I  S  I  S  T  D  T  G  S  E  R  M  K  Q

TCATACAAGCCATTAAGCAGTTCTCGCAAGTAAACGACTTCTCTTTACACTTACCTATGGACTGGACTCCAATTATTACCTCGACAATAA     900
165  I  I  Q  A  I  K  Q  F  S  Q  V  N  D  F  S  L  H  L  P  M  D  W  T  P  I  I  T  S  T  I

TTACCTTCATCACCGTCTTACTCTTCAAAAAGCAGTCCAAACTCATGTTCTCCATCATATCTTCCAGGATCATCTGGGCAACCTTGTCAA     990
195  I  T  F  I  T  V  L  L  F  K  K  Q  S  K  L  M  F  S  I  I  S  S  R  I  I  W  A  T  L  S

CTTTTTTCATCATTTGCATGATCAGTGCCTATATGTTCAACCAAATCAGGAATACCCAATTGGCAGGCGTTGGTCCTAAGGGCGAGGTTA    1080
225  T  F  F  I  I  C  M  I  S  A  Y  M  F  N  Q  I  R  N  T  Q  L  A  G  V  G  P  K  G  E  V

TGTATTTCTTGCCCAATGAATTCCAACACCAATTCGCCATTGAAACTCAAGTCATGGTTCTTATTTACGGAACATTGGCCGCGTTGGTTG    1170
255  M  Y  F  L  P  N  E  F  Q  H  Q  F  A  I  E  T  Q  V  M  V  L  I  Y  G  T  L  A  A  L  V

TCGTATTGGTCAAGGGTATACAATTCTTGCGGTCTCATTTGTATCCAGAGACCAAGAAAGCGTACTTCATTGATGCTATTTTGGCCTCCT    1260
285  V  V  L  V  K  G  I  Q  F  L  R  S  H  L  Y  P  E  T  K  K  A  Y  F  I  D  A  I  L  A  S

TTTGTGCCTTATTCATTTATGTCTTCTTTGCTGCTTTGACAACCGTGTTCACGATAAAGAGTCCTGCTTACCCTTTTCCTTTACTAAGGT    1350
315  F  C  A  L  F  I  Y  V  F  F  A  A  L  T  T  V  F  T  I  K  S  P  A  Y  P  F  P  L  L  R

TATCGGCACCATTCAAATAAGTCAGTTTTTTTTTTTGTTTTCACCATAACAAAGAGATGGAAAGTTATACTAGATAAAGATTTTTAAAAA    1440
345  L  S  A  P  F  K  *

ATATAAAAGAAAATAAAACGAAATTTATATTTTACACAGTGCATCTTGTCCCTCTCTTTCTCGCTAAAACTTAGTTTCTTCTTCGCCCAT    1530
CTCATGATTCTTCT                                                                                  1544
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Figure 1. Partial restriction endonuclease map, DNA and protein sequences, and gene disruption of the *OST3* locus. (*A*) The *OST3* locus was disrupted by replacement of the ScaI-BanI DNA fragment with the yeast integrating plasmid pRS305 carrying the *LEU2* gene. The arrows flanking the BanI site designate PCR primers used to amplify a portion of the *OST3* locus for construction of the gene disruption plasmid. Restriction sites used for constructions and mapping of the gene disruption are shown. (*B*) The nucleotide sequence of an HindIII-BspHI genomic DNA fragment containing the *OST3* gene is shown together with the predicted amino acid sequence of Ost3p. Nucleotide residues are numbered on the right; amino acid residues are numbered on the left. The termination codon is indicated by an asterisk. Solid lines beneath the protein sequence designate sequences determined by gas phase sequencing of NH_2-terminal and internal peptides. The signal peptidase cleavage site is designated by an arrow. The calculated molecular weight of 37,046 for mature Ost3p is in reasonable agreement with the M_r of 34 kD for the γ–subunit of the OST complex estimated by SDS-PAGE (Kelleher and Gilmore, 1994). Dashed underlining of the protein sequence designates four predicted membrane spanning segments (Kyte and Doolittle, 1982). A consensus site for N-linked glycosylation is enclosed in a box. These sequence data are available from the EMBL/GenBank/DDBJ under accession number U25052.

for spore viability, growth on YPD, and growth on selective media. Four segregants of the two diploid strains RGY301 and RGY302 generated by disruption of the *OST3* gene in PRY238 and YPH274, respectively, were used throughout this study (Table I).

Radiolabeling and Immunoprecipitation of Glycoproteins

Yeast cells were grown for 15–20 h at 25° or 30°C, as indicated, in synthetic minimal media (2% glucose) supplemented with the appropriate amino acids until mid log phase (A_{600} of 0.8–1.6). Yeast cells used for immunoprecipitation of invertase were transferred to minimal media containing 0.1% glucose for 20 min before labeling. Cells were collected by centrifugation, resuspended at 6 A_{600}/ml in minimal medium and incubated for an additional 15 min. Cells were labeled for the time indicated in individual figure legends with 100 μCi of [^{35}S]methionine/[^{35}S]cysteine (TRAN^{35}S; >1,000 Ci/mmol; ICN Biochemicals, Costa Mesa, CA) per A_{600} units of cells. Labeling was terminated by the addition of NaN_3 to 10 mM. To deplete lipid-linked oligosaccharides, tunicamycin was added at a concentra-

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Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
PRY238	*MATa/α, ura 3-52/ura3-52, leu2-3,112/leu2-3, 112,lys2-801/+, +/his4-619*	Orlean et al., 1988
YPH274	*MATa/α, ura3-52/ura3-52, leu2-Δ1/leu2-Δ1, lys2-801/lys2-801, ade2-101/ade2-101, trp1-Δ1/trp1-Δ1, his3-Δ200/his3-Δ200*	Sikorsky and Hieter, 1989
RGY301	*MATa/α, ura3-52/ura3-52, leu2-3,112/leu2-3,112, lys2-801/+, +/his4-619, OST3/Δost3::LEU2*	This study
RGY311	*MATα, ura3-52, leu2-3,112, lys2-801, Δost3::LEU2*	This study
RGY312	*MATa, ura3-52, leu2-3,112, his4-619*	This study
RGY313	*MATa, ura3-52, leu2-3,112, lys2-801, his4-619, Δost3::LEU2*	This study
RGY314	*MATα, ura3-52, leu2-3,112*	This study
RGY302	*MATa/α, ura3-52/ura3-52, leu2-Δ1/leu2-Δ1, lys2-801/lys2-801, ade2-101/ade2-101, trp1-Δ1/trp1-Δ1, his3-Δ200/his3-Δ200, OST3/Δost3::LEU2*	This study
RGY322	*MATa, ura3-52, leu2-Δ1, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, Δost3::LEU2*	This study
RGY323	*MATα, ura3-52, leu2-Δ1, lys2-801, ade2-101, trp1-Δ1, his3-Δ200*	This study
RGY121	*MATa, ura3-52, leu2-3,112, lys2-801, Δost1::LEU2* (pRS316*ost1-1*)	Silberstein et al., 1995
PRY98	*MATα, ura3-52, ade2-101, alg5-1*	Runge et al., 1984

tion of 10 μg/ml 15 min before radiolabeling. Rapid lysis of cells with glass beads and immunoprecipitation of radiolabeled proteins with antibodies to carboxypeptidase Y (CPY), dipeptidyl aminopeptidase B (DPAP B) and invertase were performed as described previously (Rothblatt and Schekman, 1989). Immunoprecipitated proteins were incubated for 30 min at 55°C in SDS-sample buffer, boiled for 3 min, and resolved on 8% SDS–polyacrylamide gels.

Membrane Isolation and Oligosaccharyltransferase Assay

Microsomal membranes were isolated from *Δost3*, *ost1-1*, *alg5-1* mutants and wild-type yeast grown to mid log phase at 25° or 30°C in YPD medium by a scaled-down version (Silberstein et al., 1995) of a procedure described previously (Kelleher and Gilmore, 1994). Oligosaccharyltransferase activity in digitonin extracts was assayed using an iodinated tripeptide acceptor (N^{α}-Ac-Asn-[^{125}I]Tyr-Thr-NH_2) and bovine lipid-linked oligosaccharide as a donor (Kelleher and Gilmore, 1994; Kelleher et al., 1992). The protein concentration of the microsomal membranes was determined using the Bio-Rad Protein Assay.

Immunological Methods

Equal amounts of microsomal membrane protein were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (TransBlot; Bio-Rad Laboratories, Hercules, CA). Blots were incubated for 1–16 h in TBS buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing either 1% Tween-20 or 2% nonfat dried milk plus 0.1% Tween-20 with antibodies against Ost1p, Wbp1p, Swp1p, Ost2p, or the Golgi GDPase. The preparation of antibodies to the ε subunit (Ost2p) of the oligosaccharyltransferase will be described elsewhere (Silberstein, S., P. G. Collins, D. J. Kelleher, and R. Gilmore, manuscript in preparation). Preparation and characterization of the antibody to the GDPase has been described (Berninsone et al., 1995). Antisera specific for CPY was raised in rabbits using CPY expressed in *E. coli* as the antigen as described (Raymond et al., 1992). The *E. coli* expression strain for CPY was generously provided by Dr. Tom Stevens (University of Oregon, Eugene, OR). Horseradish peroxidase–conjugated secondary antibodies were visualized using enhanced chemiluminescence (ECL Western blotting detection kit; Amersham Corp., Arlington Heights, IL). Glycoproteins were digested with endoglycosidase H (Endo H; New England Biolabs, Beverly, MA) following the manufacturer's recommendations.

Quantification of Glycosylation

The average number of oligosaccharides for each of the glycoproteins was determined by radioanalytic scanning of fluorographs using a Molecular Dynamics PhosphorImager, or by scanning ECL images using a densitometer. The ratio between the average number of oligosaccharides on a given glycoprotein in the *Δost3* mutant and the average number of oligosaccharides for that glycoprotein in a wild-type strain was expressed as the percent glycosylation relative to the wild-type.

Results

Isolation and Sequence Analysis of the OST3 Gene

The amino acid sequence of the mature NH_2 terminus of the 34-kD γ-subunit of the yeast oligosaccharyltransferase, as well as four internal tryptic peptides, was determined by gas phase sequencing. These peptide sequences were not present in the current releases of the protein sequence databanks indicating that the 34-kD subunit was a novel protein. Two pairs of degenerate oligonucleotide primers were designed based upon the sequence of two internal tryptic peptides (AFQFFQLNNVPR and LFIFKPNSPX-ILDHSXXSI), where X designates residues that could not be unambiguously assigned. PCR was used to amplify a yeast genomic DNA template. Sequencing of a primer-specific 63-bp amplification product obtained with one primer pair confirmed that we had obtained an authentic amplification product. A genomic clone encoding the 34-kD protein, henceforth, designated Ost3p, was obtained by screening a *S. cerevisiae* YEp24 library with the radiolabeled PCR product. Southern analysis of a hybridization-positive clone showed that a 2.5-kb HindIII–SphI fragment hybridized with the PCR probe.

Subsequent subcloning and sequencing revealed an open reading frame encoding a protein of 350 amino acids (Fig. 1 *B*). The predicted protein sequence contains matches for all five peptides derived from the γ-subunit of the yeast oligosaccharyltransferase. Notably, the mature NH_2-terminal methionine residue of Ost3p corresponds to residue 23 of the predicted open reading frame. Amino termini of proteins are subject to several cotranslational protein modification enzymes including signal peptidase, N^{α}-acetyltransferase and methionine aminopeptidase (Kendall et al., 1990). The specificity of modification of the amino terminus by the latter two enzymes is largely controlled by the identity of the penultimate amino acid in the protein. If Met 23 were the functional initiation codon, then the amino terminal residue of the mature Ost3 protein would be an acetylated serine rather than a methionine, based upon the experimentally determined specificity of N^{α}-acetyltransferase and methionine aminopeptidase (Kendall et al., 1990). The amino terminal sequence data is

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therefore consistent with initiation at the upstream AUG codon.

The protein sequence was analyzed for the presence of hydrophobic segments using the algorithm of Kyte and Doolittle (1982). Five hydrophobic segments were detected, the first of which resembles a typical amino terminal cleavable signal sequence (von Heijne, 1986). The location of the most probable signal peptidase processing site using the predictive method of von Heijne (1986) is in agreement with the amino terminal sequence of the mature protein. Four additional hydrophobic segments, denoted by dashed underlining in Fig. 1 *B*, are located towards the carboxy terminus of Ost3p. These four segments are predicted to function as membrane spanning domains such that the majority of the protein is located within the lumen of the endoplasmic reticulum. Although a consensus site for N-linked glycosylation is located at Asn 33, Con A blot, and Endo H digestion data indicate that Ost3p is not a glycoprotein (Kelleher and Gilmore, 1994). The potential glycosylation sequon at Asn 33 is followed by a proline residue (NKSP). Statistical analysis of utilized and nonutilized glycosylation sites in proteins that are translocated into the endoplasmic reticulum has disclosed a statistically significant bias against modification of sites followed by proline residues (Gavel and Von Heijne, 1990). Synthetic peptide substrates with proline at this position are not detectably glycosylated in vitro (Bause, 1983).

A search of protein sequence databases using the BLASTP protein sequence comparison algorithm (Altschul et al., 1990) revealed a homology between Ost3p and an open reading frame in chromosome 3 of *Caenorhabditus elegans* (P34669) that encodes a 37.7-kD protein. The *C. elegans* sequence has also been recovered as expressed sequence tags (T01933 and M88869). Despite the relatively modest sequence identity (21%) between Ost3p and the 37.7-kD *C. elegans* protein, the two proteins have an identical arrangement of four predicted membrane spanning segments. To our knowledge, vertebrate homologues of Ost3p have yet to be identified.

Disruption of the OST3 Gene

The *OST3* locus was disrupted in two diploid yeast strains as an initial step towards analyzing the function of the Ost3 protein. An 1,158-bp ScaI-BanI segment of the *OST3* gene was replaced with the yeast integrating plasmid pRS305 bearing the *LEU2* marker thereby deleting the complete open reading frame (Fig. 1 *A*). Leucine prototrophs were selected, and correct integration at the *OST3* locus was confirmed by Southern analysis (Fig. 2 *A*). An integrant from each genetic background was sporulated, asci were dissected, and spores were tested for viability on YPD plates at 30°C (Table II). Four viable spores were obtained from each tetrad, and these gave rise to four colonies of identical size indicating that the *OST3* gene is not essential for growth. Replica plating onto selective media established that the nutritional marker used for the disruption (*LEU2*) segregated 2:2 in all cases. Southern analyses of three tetrads, which was performed using a combination of restriction sites in pRS305 and DNA sequences flanking the *OST3* gene, confirmed the replacement of the *OST3* gene by pRS305 in those haploids that were leucine prototrophs. Southern analysis of the four haploid segregants of a tetrad that were used in subsequent experiments is shown in Fig. 2 *A*.

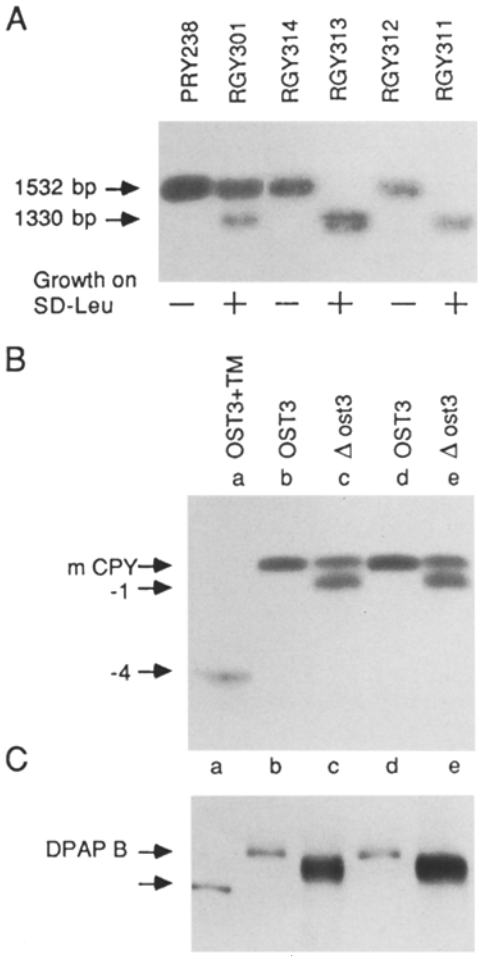

Figure 2. Disruption of the *OST3* gene and its effect upon N-linked glycosylation. (*A*) Southern blot analysis of the *OST3* disruption. Genomic DNA was isolated from PRY238, RGY301, and four haploid segregants of a RGY301 tetrad (*RGY311-RGY314*). HindIII-BspHI digests were resolved by agarose gel electrophoresis, and Southern transfers were probed with a ^{32}P-labeled HindIII-ScaI 5′ flanking fragment from the *OST3* gene. The labeled arrows designate hybridization positive bands of 1,532 and 1,330 bp that correspond to the intact and pRS305-disrupted alleles of the *OST3* gene, respectively. Disruption of the *OST3* locus was also confirmed using a ^{32}P-labeled BanI-XbaI PCR product as a 3′ flanking hybridization probe (data not shown). Growth of the six strains on synthetic minimal media lacking leucine (*SD-Leu*) is indicated below *A*. Genotypes of RGY311-RGY314 are in Table I. (*B*) The haploid segregants analyzed in *A* (*RGY311-RGY314*) were grown in minimal media at 30°C and were pulse–labeled for 30 min as described in Materials and Methods. As indicated, wild-type cells (*RGY314*) were incubated for 15 min with tunicamycin (*TM*) prior to labeling. CPY-specific immunoprecipitates from glass-bead extracts were resolved by PAGE in SDS. Fully glycosylated vacuolar CPY (*mCPY*) and underglycosylated variants of CPY are indicated by labeled arrows. (*C*) DPAP B immunoprecipitates from glass-bead extracts of cells labeled for 10 min at 30°C with TRAN^{35}S were resolved by PAGE in SDS. The migration positions of fully glycosylated DPAP B from wild-type cells and unglycosylated DPAP B from tunicamycin-treated cells are designated by arrows.

The four haploid segregants (RGY311-RGY314) de-

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Table II. Tetrad Analysis of OST3 Gene Disruption

Strain*	Genotype	Tetrads Analyzed	Viable colonies per Tetrad
PRY238	*OST3/OST3*	8	4
RGY301	*OST3/Δost3::LEU2*	8	4‡
YPH274	*OST3/OST3*	5	4
RGY302	*OST3/Δost3::LEU2*	7	4‡

*Diploid strains were sporulated, tetrads dissected on YPAD plates, and incubated for 2-3 days at 30°C. Colonies obtained were replica plated on selective medium to determine nutritional markers. PRY238 and YPH274 were the recipients for the *OST3* disruption to produce RGY301 and RGY302, respectively. Detailed genotypes for each strain are given in Table I.

‡Colonies segregated 2:2 for growth on minimal media lacking leucine.

rived from dissection of the RGY301 tetrad were then tested for differences in growth rate on solid and in liquid media at 25° and 37°C. The two haploid strains lacking the *OST3* gene (RGY311 and RGY313) did not show reduced growth rates or an altered morphology compared to the wild-type haploid segregants (RGY312 and RGY314) at either temperature. Mutations in two other subunits of the oligosaccharyltransferase cause temperature-sensitive growth defects (Silberstein et al., 1995; te Heesen et al., 1992) and extensive aggregation of cells in liquid culture (Silberstein et al., 1995).

Reduced N-linked Glycosylation Activity of the ost3 Null Mutant

Having determined that expression of the Ost3 protein is not essential for viability of *S. cerevisiae* we next asked whether asparagine-linked glycosylation was altered in an *ost3* null mutant. For this analysis we examined the glycosylation pattern of the well-characterized soluble vacuolar enzyme, carboxypeptidase Y (CPY), which is synthesized as an inactive proenzyme of 60 kD and is processed in the vacuole by cleavage of an 8-kD amino terminal propeptide (Stevens et al., 1982). Three biosynthetic forms of CPY have been identified and characterized: the 67-kD ER form with four N-linked core oligosaccharides (p1), the more extensively mannosylated 69-kD Golgi form (p2), and a proteolytically processed 61-kD vacuolar form (mature CPY). Carboxypeptidase Y was immunoprecipitated from radiolabeled cultures of the four haploid yeast strains analyzed above (RGY311-RGY314). As a control, a wild-type culture (RGY314) was treated with tunicamycin to block the assembly of the lipid-linked oligosaccharide donor (Fig. 2 *B*). Previous studies have shown that yeast cells treated with tunicamycin synthesize, transport, and proteolytically process a nonglycosylated form of CPY, albeit at a reduced rate relative to untreated cells (Stevens et al., 1982). Two haploids of the tetrad, which correspond to segregants bearing the *OST3* disruption (see Fig. 2 *A*), synthesized two major forms of mature CPY (Fig. 2 *B*). The less rapidly migrating form comigrated with vacuolar CPY synthesized by the wild-type yeast. Based upon the mobility difference between the glycosylated mature CPY and the unglycosylated vacuolar CPY synthesized by tunicamycin treated yeast (+TM), we can conclude that the more rapidly migrating form of CPY synthesized by the disruptant strains lacks one N-linked oligosaccharide chain. Essentially identical patterns of CPY glycoforms were obtained when total cell extracts from wild-type and *Δost3* mutants were analyzed by protein immunoblotting (data not shown). Endoglycosidase H digestion confirmed that the two forms differ by oligosaccharide content rather than by polypeptide mass (data not shown). Thus, the *ost3* null mutants display a readily detectable, albeit mild, reduction in glycosylation of CPY. The efficiency of utilization of the four glycosylation sequons in CPY was quantified by scanning the fluorograph shown in Fig. 2 *B*. On average, CPY synthesized by the *Δost3* mutant contains 3.5 oligosaccharides (Table III). Haploids derived from dissection of other tetrads also showed 2:2 cosegregation of the glycosylation defect and growth on minimal media lacking leucine. Analogous results were obtained when haploid segregants derived from the RGY302 strain were analyzed, indicating that underglycosylation caused by disruption of the *OST3* locus is not strain dependent.

Underglycosylation by the *ost3* null mutant was confirmed by pulse labeling studies of the vacuolar membrane protein dipeptidyl aminopeptidase B (DPAP B). DPAP B is a type II integral membrane protein with eight consensus sites for N-linked glycosylation (Roberts et al., 1989). On average, six to seven of those sites are glycosylated to yield a 110–113-kD ER form which is then converted into a mature 120-kD form by further mannose addition in the Golgi complex (Roberts et al., 1989). DPAP B was immunoprecipitated from cultures of the four haploid yeast strains (RGY311-RGY314) characterized above. As expected, the predominant glycoform of DPAP B synthesized by wild-type yeast (RGY312 and RGY314) migrated considerably slower than unglycosylated DPAP B synthesized in the presence of tunicamycin (Fig. 2 *C*). In contrast,

Table III. Relative Glycosylation of Proteins Expressed in Δost3 Mutants

Glycoprotein	Topology	Average number of sites glycosylated by wild-type yeast*	Percentage of wild-type glycosylation by *Δost3*‡	
			25°C	30°C
CPY	Soluble	4	91§	87
Invertase	Soluble	9.7	66§	ND
DPAP B	Type II membrane	6.5	ND	60§
Ost1p	Type I membrane	3.7	ND	60¶
GDPase	Type II membrane	1.5	20¶	ND
Wbp1p	Type I membrane	2	11¶	21

*See text for an explanation of the average sites glycosylated in vivo by wild-type yeast.

‡Calculated as described in Materials and Methods. (ND, not determined).

§Quantified by radioanalytic scanning of fluorographs using a Molecular Dynamics PhosphorImager.

¶Quantified by scanning an ECL image using a densitometric scanner.

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DPAP B synthesized by the *ost3* null mutant strains consisted of a diverse set of glycoforms of intermediate mobility. Despite the incomplete resolution of the intermediate glycoforms of DPAP B, underglycosylation of DPAP B by the *ost3* null mutant was clearly more severe than underglycosylation of CPY. Quantification by radioanalytic scanning indicated that DPAP B synthesized by the *ost3* null mutants contains, on average, roughly 4 N-linked oligosaccharides rather than 6.5 for the wild-type strain (Table III).

Defects in lipid-linked oligosaccharide assembly also cause reduced in vivo glycosylation of yeast glycoproteins (Huffaker and Robbins, 1983). To determine whether the reduction in glycosylation of CPY and DPAP B could be directly attributed to a defect in the oligosaccharyltransferase, we assayed digitonin-solubilized microsomal membranes for oligosaccharyltransferase activity using dolichol-linked oligosaccharide isolated from bovine pancreas as an exogenous donor and the synthetic tripeptide N^{α}-Ac-Asn-[^{125}I]Tyr-Thr-NH_2 as an exogenous oligosaccharide acceptor (Kelleher and Gilmore, 1994; Kelleher et al., 1992). Because both the lipid-linked oligosaccharide donor and the tripeptide acceptor are supplied in the in vitro assay, any in vivo defect in oligosaccharide donor assembly should not be detected using the in vitro oligosaccharyltransferase assay. Detergent extracts of membranes isolated from cultures of the two *ost3* null mutant strains (RGY311 and RGY313) exhibited a 50% reduction in oligosaccharyltransferase activity relative to the two wild-type strains (Fig. 3).

Expression and Glycosylation of the Oligosaccharyltransferase Subunits in the ost3 Null Mutant

Protein immunoblots of membranes isolated from wild-type and *Δost3* mutant strains were probed with antisera to the Ost1p, Wbp1p, Swp1p, and Ost2p to determine whether loss of Ost3p results in a reduced membrane content of the other subunits of the yeast oligosaccharyltransferase (Fig. 4). Previous studies have shown that the 64- and 62-kD glycoforms of Ost1p isolated from wild-type cells contain four and three N-linked oligosaccharides respectively (Kelleher and Gilmore, 1994; Silberstein et al., 1995). Underglycosylated forms of Ost1p were readily detected in membranes isolated from *ost3* null mutant yeast relative to the wild-type (Fig. 4 *A*). The extent of underglycosylation of Ost1p was similar to that observed for DPAP B (Fig. 2 *C* and Table III). The comparable intensity of the Ost1p band after endo H digestion suggests that microsomal membranes isolated from wild-type and *ost3* null mutants contain similar concentrations of Ost1p.

The Wbp1 protein has two consensus sites for N-linked glycosylation (te Heesen et al., 1991) both of which are used in vivo (Kelleher and Gilmore, 1994). Protein immunoblot analysis of membranes isolated from wild-type and *ost3* null mutant yeast showed that Wbp1p was severely underglycosylated in the *ost3* null mutant yeast (Fig. 4 *B* and Table III). The predominant form (~60%) of Wbp1p detected in membranes from the *ost3* null mutant comigrated with Endo H digested Wbp1p. A comparison of the intensity of the Endo H digested samples suggests that the membrane content of Wbp1p is not reduced in the *ost3* null mutant strain. As observed for Ost1p and Wbp1p, a reduction in the membrane content of Swp1p and Ost2p was not apparent in the *Δost3* mutant yeast (Fig. 4, *C* and *D*). Together, these results suggest that loss of Ost3p does not lead to a reduced synthesis, membrane incorporation or stability of any of the known subunits of the oligosaccharyltransferase complex, but instead reduces the in vivo and in vitro activity of the oligosaccharyltransferase.

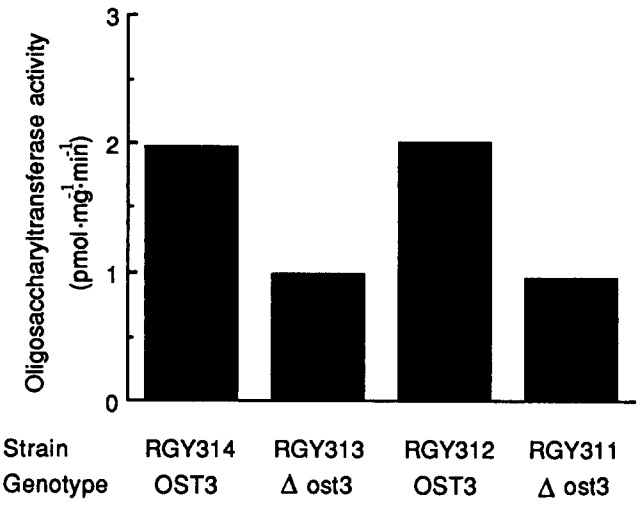

Figure 3. In vitro oligosaccharyltransferase activity of *ost3* null mutants. Microsomal membranes were isolated from cultures of two wild-type (*RGY312* and *RGY314*) and two *Δost3* mutants (*RGY311* and *RGY313*) after growth in YPD media at 30°C. The oligosaccharyltransferase activity of digitonin extracts was assayed in vitro as described in the Materials and Methods. Specific activity values ($pmol \cdot mg^{-1} \cdot min^{-1}$) are the average of two determinations.

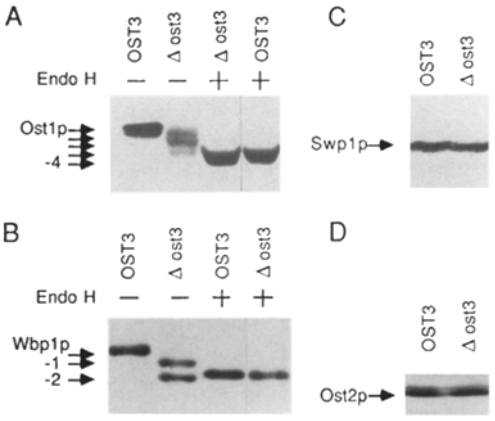

Figure 4. Glycosylation and expression of four subunits of the yeast oligosaccharyltransferase complex. Microsomal membrane proteins that were isolated from wild-type (*RGY314*) and *Δost3* mutant (*RGY313*) cells were resolved by SDS-PAGE and transferred to PVDF membranes. Each gel lane contained the following amounts of membrane protein: Ost1p (40 μg), Wbp1p (5 μg), Swp1p (60 μg), and Ost2p (60 μg). As indicated, the membrane proteins were treated with Endo H prior to SDS-PAGE analysis. Antibodies specific for Ost1p (*A*), Wbp1p (*B*), Swp1p (*C*), or Ost2p (*D*) were used as probes and visualized as described in the Materials and Methods. Fully glycosylated, underglycosylated and deglycosylated forms of Ost1p (*A*) and Wbp1p (*B*) are indicated by labeled arrows.

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Selective Glycosylation Deficiency of the Δost3 Mutant

The different glycoproteins analyzed in the preceding experiments showed considerable variability in the extent of underglycosylation, ranging between 21% of wild-type glycosylation for Wbp1p to 87% of wild-type for CPY (Table III). We next asked whether this variability in the extent of underglycosylation was peculiar to the *ost3* mutant, or was instead a general property of yeast with defects in N-linked glycosylation. For the following experiments we compared the *ost3* null mutant to two other yeast mutants deficient in asparagine-linked glycosylation; *alg5-1* (Huffaker and Robbins, 1983; te Heesen et al., 1994) and *ost1-1* (Silberstein et al., 1995). The *alg5-1* mutant accumulates the lipid-linked oligosaccharide $Man_9GlcNAc_2$-P-P-dolichol as the largest oligosaccharide donor, due to a deficiency in UDP-glucose/dolichyl-phosphate glucosyltransferase (Huffaker and Robbins, 1983; te Heesen et al., 1994). Like the *ost3* null mutant, *alg5-1* yeast do not show a growth defect at 25° or 37°C (Runge et al., 1984), but do synthesize underglycosylated forms of invertase (Huffaker and Robbins, 1983) and CPY (Stagljar et al., 1994; te Heesen et al., 1994). Synthesis of underglycosylated proteins by the *alg5-1* mutant can be ascribed to the preference of the oligosaccharyltransferase for the fully assembled dolichol-linked oligosaccharide donor (Trimble et al., 1980). When grown at the permissive temperature (25°C), *ost1-1* cells show reduced transfer of core oligosaccharides to soluble and membrane-bound glycoproteins, yet grow at a wild-type rate (Silberstein et al., 1995).

The extent of underglycosylation of CPY by the *alg5-1* and *ost1-1* mutant yeast was quite similar; in both cases the predominant glycoform lacks one N-linked oligosaccharide (Fig. 5 *A*). Thus, underglycosylation of CPY by the *alg5-1* and *ost1-1* mutants was more pronounced than that observed for the *ost3* null mutant. Invertase was examined as a second soluble glycoprotein (Fig. 5 *B*). Three major forms of invertase can be identified when yeast cells induced to synthesize invertase are labeled for a brief time period (Esmon et al., 1981). Both the 60-kD cytoplasmic form of invertase and unglycosylated invertase synthesized in the presence of tunicamycin (Fig. 5 *B*, lane *b*) are readily resolved from several core-glycosylated ER glycoforms of invertase (Fig. 5 *B*, lane *a*). The predominant glycoform of invertase secreted by wild-type cells contains, on average, 9–10 N-linked oligosaccharides (Reddy et al., 1988). Mature invertase migrates as a 100–150-kD smear due to extensive elongation of core oligosaccharide chains in the Golgi apparatus (Esmon et al., 1981). Essentially identical distributions of underglycosylated forms of invertase were observed when two *ost3* null mutants (RGY311 and RGY322) were analyzed (Fig. 5 *B*, lanes *c* and *d*). As RGY322 is a haploid derived from a different diploid strain (YPH274), we can conclude that disruption of *OST3* in two different genetic backgrounds yields a similar underglycosylation phenotype. The ER form of invertase synthesized by *alg5-1* cells contains, on average, 3.7 less N-linked oligosaccharides than invertase synthesized by a wild-type strain (Fig. 5 *B*). When invertase was immunoprecipitated from *Δost3*, and *ost1-1* mutant cells grown at 25°C, the ER form of invertase respectively lacked, on average, 3.3 and 3.0 oligosaccharides relative to the wild-type

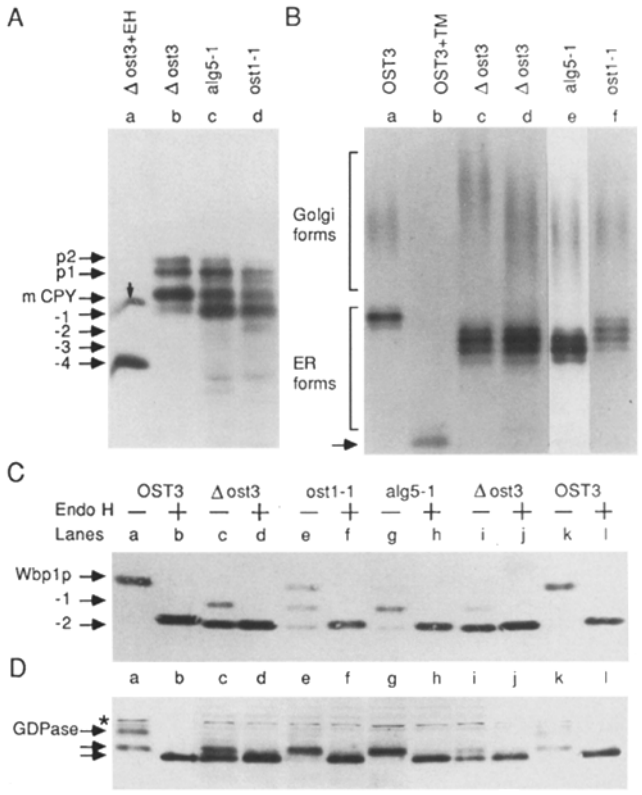

Figure 5. Selective underglycosylation of Wbp1p and the GDPase. Wild-type RGY312 (*B*, lanes *a* and *b*), mutant *Δost3* (RGY311, *A*, lanes *a* and *b*, *B*, lane *c* and RGY322, *B*, lane *d*), *alg5-1* or *ost1-1* cells were grown in minimal media at 25°C. (*A*) CPY-specific immunoprecipitates from cells labeled for 30 min were resolved by PAGE in SDS. The sample in lane *a* was incubated with Endo H (*EH*) prior to loading. Fully glycosylated and underglycosylated forms of CPY are indicated by labeled arrows. The vertical arrowhead in the Endo H treated sample designates deglycosylated proCPY. (*B*) Yeast cells induced to express invertase (see Materials and Methods) were pulse labeled for 10 min, and invertase immunoprecipitates were resolved by SDS-PAGE. Wild type (RGY312) cells were incubated for 15 min with tunicamycin prior to labeling. The ER and Golgi forms of invertase are indicated by labeled brackets. The unglycosylated cytoplasmic form of invertase, indicated by an arrow, was not precipitated efficiently. (*C* and *D*) Microsomal membranes were isolated from 25°C cultures of wild-type (RGY312, lanes *a* and *b*, and RGY323, lanes *k* and *l*), *Δost3* (RGY311, lanes *c* and *d*, and RGY322, lanes *i* and *j*), *ost1-1*, and *alg5-1* yeast. After treatment with Endo H, as indicated, the proteins were resolved by SDS-PAGE and immunoblots were probed with antibodies specific for Wbp1p (*C*) or GDPase (*D*). The regions of the immunoblots that contain fully glycosylated and underglycosylated forms of Wbp1p and GDPase are indicated by clusters of arrows. Each gel lane for the GDPase blot contained 40 μg of membrane protein. The band indicated by the asterisk (*) is a nonspecific unglycosylated protein.

strain (Fig. 5 *B* and Table III). Thus, the extent of underglycosylation by *alg5-1*, *ost1-1*, and *Δost3* was fairly similar when invertase was used as the test glycoprotein.

Membranes that were isolated from 25°C cultures of *Δost3*, *ost1-1*, *alg5-1* and two wild-type control strains were subjected to protein immunoblot analysis using antibodies to Wbp1p (Fig. 5 *C*). As observed previously (Silberstein et al., 1995), glycosylation of Wbp1p is reduced in the *ost1-1*

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mutant relative to a wild-type control strain. The Wbp1p glycoforms detected in the *ost1-1* and *alg5-1* membranes contain, on average, 1.1 and 0.9 oligosaccharides, respectively. In contrast, the predominant form of Wbp1p synthesized by both *Δost3* strains lacks both oligosaccharides (Table III). Since Wbp1p is a subunit of the oligosaccharyltransferase, we evaluated the glycosylation of an unrelated Golgi membrane protein, the GDPase, which contains three consensus sites for N-linked glycosylation (Abeijon et al., 1993; Berninsone et al., 1995). Two discrete GDPase glycoforms were observed in both wild-type strains (Fig. 5 *D*, lanes *a* and *k*), indicating that the oligosaccharides on the GDPase are not subject to extensive outer chain elongation. Based upon the mobility increase caused by Endo H digestion, we tentatively conclude that one of the three consensus sites in the GDPase is not modified in vivo, while a second site is subject to partial glycosylation. One of the consensus sites in the GDPase is located 17 residues from the signal–anchor sequence in this type II membrane protein (Abeijon et al., 1993). Although the canine OST has been shown to modify consensus sites within 13 residues of a membrane-spanning segment (Nilsson and von Heijne, 1993), comparable experiments have not been performed with yeast membranes. The predominant form (~80%) of the GDPase detected in membranes prepared from both *ost3* null mutant strains was not glycosylated (Fig. 5 *D*, lanes *c* and *i*; Table III). In contrast, the predominant form of the GDPase synthesized by the *alg5-1* and *ost1-1* mutants contained a single oligosaccharide. Hence, the GDPase, like Wbp1p, is more severely underglycosylated by the *ost3* null mutant than by two other yeast strains that have lesions in the assembly (*alg5-1*) or transfer (*ost1-1*) of N-linked oligosaccharides.

Discussion

We have isolated and characterized the *S. cerevisiae OST3* gene that encodes a 34-kD protein from yeast oligosaccharyltransferase preparations obtained using three different procedures (Kelleher and Gilmore, 1994; Knauer and Lehle, 1994; Pathak et al., 1995). However, the Ost3 protein may be present in substoichiometric amounts compared to the other OST subunits as judged by a reduced relative staining intensity with silver or Coomassie blue (Kelleher and Gilmore, 1994; Pathak et al., 1995). Additional studies will be required to address subunit stoichiometry in the intact endoplasmic reticulum membrane, and to determine whether the Ost3 protein is an accessory or a regulatory subunit. Expression of the *OST3* gene is required for normal glycosylation of each of the six glycoproteins that we have tested. While underglycosylation of proteins in vivo can be caused by several mechanisms, the reduced in vitro oligosaccharyltransferase activity of the *Δost3* mutant is most readily explained by a lesion in the oligosaccharyltransferase. Since none of the other OST subunits was present in reduced amounts in membranes prepared from the *Δost3* mutant, we conclude that Ost3p is not required for synthesis, assembly or stability of the OST complex.

The Ost3 protein sequence does not show homology to the sequence of any of the three subunits of the mammalian oligosaccharyltransferase, nor does it appear to be related to any of the previously characterized subunits of the yeast OST. The existence of a potential homologue in *C. elegans* raises the possibility that vertebrate organisms might contain a related protein. As noted previously, the purified yeast oligosaccharyltransferase is roughly fivefold more active than the heterotrimeric canine OST complex (Kelleher and Gilmore, 1994). Based upon the in vitro OST assays described here, we would anticipate that dissociation of a putative canine Ost3p homologue during purification would lead to a reduction in OST activity. Alternatively, a putative Ost3 protein in a vertebrate organism might act as a stimulatory factor that is not stably associated with the oligosaccharyltransferase. Studies to differentiate between these alternatives using canine microsomes are in progress.

Ost3 null mutant yeast are able to grow at 25°, 30°, or 37°C without showing a detectable reduction in growth rate relative to appropriate control strains. This observation was unexpected, as the genes encoding the three previously characterized subunits of the yeast oligosaccharyltransferase are all essential (Silberstein et al., 1995; te Heesen et al., 1992, 1993). When we evaluated glycosylation of secretory and membrane proteins in vivo, the *Δost3* strains exhibited underglycosylation at 25° (Fig. 5), 30° (Figs. 2 and 4), and at 37°C (data not shown). Thus, even at elevated temperatures, yeast cells will tolerate considerable underglycosylation of proteins. In this regard, *Δost3* mutants are phenotypically similar to a subset of the *alg* mutants (e.g., *alg5, alg6,* and *alg8*) that effect late stages in assembly of the lipid-linked oligosaccharide, underglycosylate proteins, yet are viable at 37°C (Huffaker and Robbins, 1983).

Interestingly, glycosylation of proteins was differentially reduced upon disruption of the *OST3* gene. The extent of underglycosylation, relative to wild-type, ranged between 13% for CPY and 79% for Wbp1p (Table III). Although fewer glycoproteins were examined in previous characterizations of oligosaccharyltransferase mutants (Silberstein et al., 1995; te Heesen et al., 1992, 1993), a pronounced bias against glycosylation of specific substrates was not reported. As this property was not anticipated, we directly compared the *Δost3* mutant with a second oligosaccharyltransferase mutant (*ost1-1*) and a yeast strain bearing a defect in lipid-linked oligosaccharide assembly (*alg5-1*). Previous results indicate that *ost1-1* mutants are defective in core oligosaccharide transfer to CPY, DPAP B, and Wbp1p (Silberstein et al., 1995). We confirmed and extended those results using invertase and the Golgi GDPase as additional test substrates. As observed here, yeast that are defective in donor assembly (*alg5-1*) or oligosaccharide transfer (*ost1-1*) underglycosylate all glycoproteins tested, without showing a dramatic bias against selected acceptor substrates (e.g., Wbp1p and GDPase). Control experiments indicate that the observed bias in glycosylation of some proteins is not a trivial artifact caused by differences in growth temperature, genetic background or detection method, but is instead an inherent property of all *Δost3* mutants isolated in this study. However, it would be premature to conclude that biased underglycosylation will not be displayed by any other mutant that affects the donor assembly pathway or the oligosaccharyltransferase.

Due to the limited number of glycoproteins analyzed, we can not draw an unequivocal conclusion concerning

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what feature or features of the acceptor substrates are responsible for biased underglycosylation. However, we considered several properties of the nascent glycoproteins that might contribute to underglycosylation of a subset of substrates. Analysis of 427 used glycosylation sites has revealed that N-X-T sites are roughly twice as common as N-X-S sites, while these two sequences occur with similar frequency in cytosolic proteins, or non-used consensus sequences within secreted proteins (Gavel and Von Heijne, 1990). In vitro assays using synthetic peptides indicate that N-X-T peptides are more rapidly glycosylated than N-X-S peptides (Bause, 1984). Could biased underglycosylation by *Δost3* mutants be explained by reduced recognition of one type of acceptor sequence? The observed twofold reduction in the in vitro OST activity is not consistent with a N-X-S consensus site-specific lesion given that the assay substrate is N^{α}-Ac-Asn-[^{125}I]Tyr-Thr-NH_2. The two proteins that were most heavily underglycosylated (Wbp1p and GDPase) each contain one N-X-S site, while CPY lacks N-X-S sites. However, the relatively modest underglycosylation of invertase, which contains three N-X-S sites out of thirteen total sites, does not provide strong support for the hypothesis that *Δost3* mutants are selectively deficient in glycosylation of N-X-S sites. A thorough analysis of the carbohydrate content of each of the glycosylation sequons in invertase has revealed that seven sites are modified in all invertase molecules synthesized by wild-type yeast, while five additional consensus sites are subject to variable glycosylation ranging between 30 and 80% (Reddy et al., 1988). We have not determined which sites in invertase are subject to underglycosylation by the *Δost3* mutant, so it is not clear whether the reduction in glycosylation is a consequence of reduced modification at all normally used sites, or is instead due to selective underglycosylation of a subset of consensus sites.

Perhaps the most obvious structural difference between the marginally and severely underglycosylated proteins was that the latter category consists of membrane proteins while the former category is comprised primarily of soluble proteins. Whether or not this correlation is significant, the fundamental question to be addressed is how or why the *Δost3* mutant exhibits biased underglycosylation of certain proteins (i.e., Wbp1p and the GDPase). Although membrane proteins appear to be slightly more prone to underglycosylation when the oligosaccharyltransferase is impaired (Silberstein et al., 1995), the biased underglycosylation of membrane proteins by the *Δost3* mutant cannot be readily explained by a reduced catalytic efficiency of the oligosaccharyltransferase. How might the Ost3 protein enhance glycosylation of certain classes of nascent glycoproteins? Perhaps the Ost3 protein interacts with subunits of the yeast translocon to localize the oligosaccharyltransferase in the vicinity of a translocation site. Protein translocation across the yeast endoplasmic reticulum can occur via a cotranslational signal recognition particle (SRP)-dependent pathway, or a posttranslational chaperone dependent pathway (for a review see Sanders and Schekman, 1992). Interestingly, translocation or integration of some proteins is most severely affected by lesions in SRP or SRP receptor (Hann and Walter, 1991; Ogg et al., 1992), while translocation of other proteins is most severely impaired by mutations in the components of the Sec63p complex (Feldheim et al., 1993; Stirling et al., 1992). Notably, translocation of CPY is not affected in a *ΔSRP54* yeast strain, in contrast to integration of DPAP B which is impaired (Hann and Walter, 1991; Ogg et al., 1992). Yeast bearing mutations in the Sec63p complex are markedly deficient in translocation of CPY, yet show essentially wild-type integration of DPAP B (Stirling et al., 1992). Taken together, these results suggest that different proteins are translocated through Sec61p translocation channels (Müsch et al., 1992; Sanders et al., 1992) that have different auxiliary components that are specific for the cotranslational or posttranslational targeting pathway (Hann and Walter, 1991). Conceivably, biased underglycosylation in the *Δost3* yeast may be due to an impaired interaction between the oligosaccharyltransferase and the auxiliary proteins that comprise the SRP-dependent subset of Sec61p translocation channels. Alternatively, the Ost3 protein may enhance glycosylation of some acceptor substrates by increasing the time window that the nascent polypeptide is in contact with the oligosaccharyltransferase prior to the initiation of folding reactions that will render unused glycosylation sites refractory to modification.

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